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Description

This invention relates to novel human tumor necrosis factor (hereinafter referred to as TNF) polypeptide mutants and DNAs encoding these mutants.

TNF is a physiologically active substance discovered by Carswell et al. in 1975 [Proc. Natl. Acad. Sci., USA, 72, 3666 (1975)]. It is characterized by showing strong cytotoxic activity against tumor cells in vitro and necrotizing a transplanted tumor in vivo [L. J. Old, Cancer Res., 41, 361 (1981)].

In 1984 to 1985, DNAs encoding rabbit, human and mouse TNFs were isolated [European Patent Publication No. 146026, European Patent Publication No. 155549, European Patent Publication No. 158286 and Fransen et al., Nucleic Acids Res., 13, 4417 (1985)], and the entire primary structures of their TNF polypeptides were elucidated.

Isolation of DNAs encoding TNFs, particularly DNA encoding human TNF, enabled human TNF to be produced in microorganisms by genetic engineering techniques, and various properties of human TNF were studied in greater detail. These studies led to the determination that human TNF has strong cytotoxic activity in vitro and antitumor activity in vivo [D. Pennica et al., Nature, 312, 724 (1984); T. Shirai et al., Nature, 313, 803 (1985); and M. Yamada et al., J. Biotechnology, 3, 141 (1985)].

Studies on the mutation of human TNF polypeptides have also been undertaken, and several patents have been published (PCT International Patent Publication No. WO86/02381, PCT International Patent Publication No. WO86/04606, European Patent Publication No. 168214, European Patent Publication No. 155549 which corresponds to U. S. Patent Application Serial No. 708846, and Japanese Patent Publication No. 48632/1987). The first three patents merely refer to the mutation of human TNF polypeptide composed of 157 amino acids or give a disclosure of specific mutations. The remaining two patents disclose or refer to the mutation of human TNF polypeptide composed of 155 amino acids. The human TNF polypeptide mutants of the present invention, however, differ in amino acid sequence from the mutants specifically disclosed in these patents.

The present inventors actually mutated amino acid(s) in the amino acid sequence of human TNF polypeptide composed of 155 amino acid residues and polypeptides resulting from deletion of amino acid-(s) beginning with the N-terminus of the human TNF polypeptide, and examined the properties of the resulting human TNF polypeptide mutants. This work has led to the discovery that soluble polypeptides can be obtained only when specific amino acid(s) at specific site(s) in the above polypeptides are mutated. It is an object of this invention, therefore, to provide a group of soluble human TNF polypeptide mutants.

Another object of this invention is to provide human TNF polypeptide mutants which are soluble and have TNF activity.

It has also been found that other specific mutants in the above group surprisingly show excellent antitumor activity in vivo despite their very low cytotoxic activity in vitro, and that in these mutants, pyrogenicity which is undesirable for use as pharmaceuticals is considerably reduced. It is a further object of this invention therefore to provide human TNF polypeptide mutants which show very low cytotoxic activity in vitro, but excellent antitumor activity in vivo, and have reduced side-effects. The fact that these mutants show excellent activity in vivo despite their low activity in vitro indicates that structures (active centers) essential to cytotoxic activity in vitro and antitumor activity in vivo which are typical biological activities of TNF do not always exist at the same site in TNF polypeptide molecule. This also leads to the presumption that active centers of various biological activities of TNF differ from one another.

Further objects of this invention will become apparent from the following description.

To simplify the description, the following abbreviations are used in the present specification and claims.

A:	adenine
C:	cytosine
G:	guanine
T:	thymine
Ala:	alanine
Arg:	arginine
Asn:	asparagine
Asp:	aspartic acid
Cys:	cysteine
Gln:	glutamine
Glu:	glutamic acid
Gly:	glycine
His:	histidine
Ile:	isoleucine

Leu:	leucine
Lys:	lysine
Met:	methionine
Phe:	phenylalanine
Pro:	proline
Ser:	serine
Thr:	threonine
Trp:	tryptophan
Tyr:	tyrosine
Val:	valine
DNA:	deoxyribonucleic acid
cDNA:	complementary DNA
dATP:	deoxyadenosine triphosphate
dCTP:	deoxycytidine triphosphate
dGTP:	deoxyguanosine triphosphate
dTTP:	deoxythymidine triphosphate
kbp:	kilo base pairs
bp:	base pairs
SDS:	sodium dodecylsulfate
MW:	molecular weight;
KD:	kilodaltons
SD sequence:	Shine-Dalgarno sequence
Meth A sarcoma:	methylcholanthrene-induced sarcoma

In the present specification, the base sequence shown by a single strand is the base sequence of a sense strand, and the left end is a 5'-terminus and the right end, a 3'-terminus. In the amino acid sequence, the left end is an N-terminus, and the right end, a C-terminus.

Fig. 1 shows the restriction endonuclease mapping of cloned cDNA encoding human TNF;

Figs. 2 and 3 show the steps of constructing an expression plasmid pHNY-32 (in Example 1);

Fig. 4 shows the steps of preparing a PL-DNA fragment for construction of an expression plasmid pHPL-115 (in Example 2);

Fig. 5 shows an elution pattern by high performance liquid chromatography of peptide fragments from polypeptide TNF-115L by digestion with lysyl endopeptidase (in Example 2);

Fig. 6 shows the steps of constructing an expression plasmid pHTR91 (in Referential Example 1).

The present invention relates to a polypeptide having an amino acid sequence represented by formula [I]

Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro
Val Ala His Val Val Ala Asn Pro Gln Ala
Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
Ala Asn Ala Leu Leu Ala Asn Gly Val Glu
Leu Arg Asp Asn Gln Leu Val Val Pro Ser
Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val
Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr
His Val Leu Leu Thr His Thr Ile Ser Arg
Ile Ala Val Ser Tyr Gln Thr Lys Val Asn
Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln
Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly
Val Phe Gln Leu Glu Lys Gly Asp Arg Leu
Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu
Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe
Gly Ile Ile Ala Leu ... [I]

in which at least one of
the following replacements of amino acids is effected:
31st Ala by Thr,
32nd Asn by Ala, Cys, Asp, His, Ile, Arg, Ser, Thr, Val or Tyr,
115th Pro by Ser, Ala, Phe, Asn, Gly, Tyr, Val, Glu, Met, Ile, Asp, Trp, Leu or Lys,
and
117th Tyr by His.
Especially preferred mutants are polypeptides having the amino acid sequence of formula [I] in which
32nd Asn is replaced by Tyr, His, Asp or Ser,
115th Pro is replaced by Leu, Ser, Asp or Gly,
or
117th Tyr is replaced by His.

The present invention also relates to DNAs encoding the above polypeptide mutants of this invention.

The DNAs encoding the novel human TNF polypeptide mutants of the invention can be produced by preparing DNA encoding human TNF or its precursor by a known method, such as the method described in European Patent Publication No. 155549 or a method of chemical synthesis, and then preparing DNAs encoding the above mutants by point mutation of the resulting DNA in accordance with the method of Wang et al. [Science, 224, 1431 (1984)], or preparing DNAs encoding the above mutants by partial replacement of the resulting DNA using suitable restriction endonucleases and synthetic oligodeoxyribonucleotide adapters in which the base sequence at the desired site(s) is artificially altered.

For example, DNA encoding a polypeptide mutant of formula [I] in which the 115th amino acid (Pro) is replaced by Leu can be produced by the following procedure.

DNA having a base sequence encoding human TNF precursor is isolated by the method described in European Patent Publication No. 155549. The base sequence of DNA encoding the human TNF precursor is shown in Table 8 attached, and a sequence from the 235th base to the 699th base in this base sequence corresponds to a base sequence encoding human TNF. The codon encoding the 115th amino acid (Pro) in the amino acid sequence of human TNF corresponds to the 577th to 579th bases (CCC) in Table 8. A DNA fragment containing this codon is cut out with a combination of suitable restriction endonucleases. Separately, a DNA fragment containing a base sequence resulting from replacing the codon (CCC) for Pro in the above DNA fragment by codon (CTC) for Leu is chemically synthesized. By substituting the synthesized DNA fragment for the cut out DNA fragment, DNA encoding the above polypeptide mutant can be produced.

More specifically, A DNA fragment corresponding to the 555th to 603rd bases in Table 8 is cut out by using restriction endonucleases DdeI and PvuII, for example.

On the other hand, the oligodeoxyribonucleotide adapters having the following base sequences are chemically synthesized.

```
5'-TGAGGCCAAGCCCTGGTATGAGCTCAT-3'
3'-CCGGTTCGGGACCATACTCGA-5'
```

and

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5'-CTATCTGGGAGGGGTCTTCCAG-3'
3'-GTAGATAGACCCTCCCCAGAAGGTC-5'
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The resulting DNA adapters are substituted for the cut out DNA fragment corresponding to the 555th to 603rd bases in Table 8.

By inserting the DNA into a suitable expression vector so that it has a suitable sequence, introducing the vector into a suitable host, and culturing the resulting transformant by techniques known in the art, human TNF polypeptide mutants of the invention can be produced. More specifically, an expression vector for production of the polypeptide mutant of the invention can be produced by preparing a DNA fragment having a translation initiation codon ATG at the 5'-terminus and a termination codon at the 3'-terminus in the DNA having a base sequence encoding the polypeptide mutant itself of the invention, joining the DNA fragment following a suitable promoter and the SD sequence, and then inserting the resulting fragment into a vector. Examples of the promoter are lac, trp, tac, phoS, phoA, PL, and SV40 early promoter. Examples of the vector are plasmids (e.g., pBR322), phages (e.g., lambda phage derivatives), and viruses (e.g., SV40). Transformant can be obtained by introducing the resulting expression vector for production of the polypeptide mutant of the invention into a suitable host, for example, E. coli, by the method of Cohen et al. [Proc. Natl. Acad. Sci., USA, 69, 2110 [1972)]. Then, by culturing the transformant under suitable culturing conditions, the desired polypeptide mutant or one in which Met is joined to its N-terminus can be produced. The cultured cells are treated by, for example, lysozyme digestion, freeze-thawing, ultrasonic rupture, or by using a French press, and then centrifuged or filtered to obtain an extract containing the polypeptide mutant of the invention. The desired polypeptide mutant can be isolated by purifying the extract in accordance with a general method of purifying proteins (such as ultra-filtration, dialysis, ion exchange chromatography, gel filtration, electrophoresis and affinity chromatography).

The reaction of an organic or inorganic acid or a base with the polypeptide mutant of the invention can give its salt.

Human TNF polypeptide mutants of the invention will be described below in more detail with reference to experimental examples.

(1) Various human TNF polypeptide mutants were produced by culturing the transformants obtained in Examples and Referential Examples given hereinbelow.

Specifically, the transformants were cultured by the method shown in Example 1-(2), and the various human TNF polypeptide mutants produced in the E. coli cells were extracted into 50 mM Tris-HCl buffer (pH 8) containing 0.1% lysozyme and 30 mM NaCl.

The amounts of the desired human TNF polypeptide mutants recovered in the extracts were measured by EIA (enzyme immunoassay) as the amount of the polypeptide which reacted immunologically with an anti-human TNF antibody. The method of determination of the human TNF polypetide mutant according to EIA is based on the following principle.

A competitive binding reaction for an anti-human TNF rabbit antiserum was done between the human TNF polypeptide mutant in an assay sample and human TNF labelled with beta-galactosidase. Then, by adding anti-rabbit IgG goat antiserum insolubilized by binding to a bacterial cell wall, a complex of enzyme-labelled human TNF/anti-human TNF rabbit antibody/anti-rabbit IgG goat antibody was formed. The reaction mixture was centrifuged to obtain a solid phase. The amount of the enzyme-labelled human TNF in the above complex which was recovered in the solid phase was determined by using its enzyme activity as an index.

Specifically, 2-nitrophenyl-beta-D-galacto-pyranoside was used as an enzyme substrate, and the amount of the digested product (2-nitrophenol) of the substrate formed by the enzyme reaction was

determined by the absorbance at a wavelength of 410 nm. The amount of the enzyme-labelled human TNF in the complex reflects the amount of the human TNF polypeptide mutant in the assay sample.

The amount of the human TNF polypeptide mutant in the assay sample was determined by using a standard curve prepared separately by using human TNF.

In the preparation of the anti-human TNF rabbit antiserum, pure human TNF produced by the method of Yamada et al. [J. Biotechnology, 3, 141 (1985)] was used as an antigen.

The results are shown in Table 3.

When the amount of the desired human TNF polypeptide mutant detected in the cell extract is nearly comparable to that of human TNF used as a control, the solubility of the polypeptide mutant is expressed as (++). Its solubility is expressed as (+) when its detected amount is smaller than the control, and as (-) when it is much smaller than the control or the desired polypeptide mutant is not detected.

It is presumed that the polypeptide mutants having solubilities expressed as (-) underwent structural change and thus markedly decreased in solubility, or were unstable in the E. coli cells.

Table 3

Solubilities of Human TNF Polypeptide Mutants:

Poly-peptide mutant	Mutation: position		solubility
TNF-12T	12th	Ala→Thr	(-)
TNF-13Y	13th	His→Tyr	(-)
TNF-14A	14th	Val→Ala	(-)
TNF-16V	16th	Ala→Val	(++)
TNF-17T	17th	Asn→Thr	(+)
TNF-24F	24th	Leu→Phe	(+)
TNF-26R	26th	Trp→Arg	(-)
TNF-31T	31st	Ala→Thr	(++)
TNF-32A	32nd	Asn→Ala	(++)
TNF-32C	32nd	Asn→Cys	(++)
TNF-32D	32nd	Asn→Asp	(++)
TNF-32H	32nd	Asn→His	(++)
TNF-32I	32nd	Asn→Ile	(++)
TNF-32R	32nd	Asn→Arg	(++)
TNF-32S	32nd	Asn→Ser	(++)
TNF-32T	32nd	Asn→Thr	(++)
TNF-32V	32nd	Asn→Val	(++)
TNF-32Y	32nd	Asn→Tyr	(++)
TNF-32G	32nd	Asn→Gly	(++)
TNF-32L	32nd	Asn→Leu	(++)
TNF-34I	34th	Leu→Ile	(++)
TNF-35P	35th	Leu→Pro	(-)
TNF-36V	36th	Ala→Val	(++)
TNF-44D	44th	Asn→Asp	(-)
TNF-45P	45th	Gln→Pro	(-)
TNF-48M	48th	Val→Met	(++)
TNF-50P	50th	Ser→Pro	(-)
TNF-54C	54th	Tyr→Cys	(-)
TNF-54H	54th	Tyr→His	(-)

- to be continued -

Table 3 (continued)

Poly-peptide mutant	Mutation: position		solubility
TNF-58P	58th	Ser→Pro	(-)
TNF-59L	59th	Gln→Leu	(-)
TNF-60D	60th	Val→Asp	(-)
TNF-60G	60th	Val→Gly	(-)
TNF-62S	62nd	Phe→Ser	(-)
TNF-67S	67th	Cys→Ser	(++)
TNF-70Y	70th	Thr→Tyr	(++)
TNF-73P	73rd	Leu→Pro	(++)
TNF-82D	82nd	Ala→Asp	(++)
TNF-85H	85th	Tyr→His	(++)
TNF-89I	89th	Val→Ile	(++)
TNF-93P	93rd	Ser→Pro	(-)
TNF-94T	94th	Ala→Thr	(++)
TNF-97N	97th	Ser→Asn	(++)
TNF-98H	98th	Pro→His	(++)
TNF-98L	98th	Pro→Leu	(++)
TNF-99S	99th	Cys→Ser	(++)
TNF-103P	103rd	Thr→Pro	(++)
TNF-113C	113th	Tyr→Cys	(++)
TNF-115H	115th	Pro→His	(++)
TNF-115Q	115th	Pro→Gln	(++)
TNF-115S	115th	Pro→Ser	(++)
TNF-115A	115th	Pro→Ala	(++)
TNF-115F	115th	Pro→Phe	(+)
TNF-115N	115th	Pro→Asn	(++)
TNF-115T	115th	Pro→Thr	(++)
TNF-115G	115th	Pro→Gly	(++)
TNF-115Y	115th	Pro→Tyr	(++)
TNF-115V	115th	Pro→Val	(++)
TNF-115E	115th	Pro→Glu	(++)

- to be continued -

Table 3 (continued)

Poly-peptide mutant	Mutation: position		solubility
TNF-115M	115th	Pro→Met	(+)
TNF-115I	115th	Pro→Ile	(++)
TNF-115D	115th	Pro→Asp	(++)
TNF-115W	115th	Pro→Trp	(++)
TNF-115L	115th	Pro→Leu	(++)
TNF-115K	115th	Pro→Lys	(++)
TNF-115R	115th	Pro→Arg	(+)
TNF-117H	117th	Tyr→His	(++)
TNF-118Q	118th	Leu→Gln	(++)
TNF-121G	121st	Val→Gly	(-)
TNF-124Q	124th	Leu→Gln	(-)
TNF-128A	128th	Asp→Ala	(-)
TNF-128N	128th	Asp→Asn	(-)
TNF-131I	131st	Ser→Ile	(++)
TNF-132T	132nd	Ala→Thr	(++)
TNF-135D	135th	Asn→Asp	(+)
TNF-138Y	138th	Asp→Tyr	(+)
TNF-141Y	141st	Asp→Tyr	(++)
TNF-143V	143rd	Ala→Val	(++)
TNF-144K	144th	Glu→Lys	(++)
TNF-145C	145th	Ser→Cys	(++)
TNF-146E	146th	Gly→Glu	(++)
TNF-148D	148th	Val→Asp	(-)
TNF-148G	148th	Val→Gly	(-)
TNF-150L	150th	Phe→Leu	(-)
TNF-151E	151st	Gly→Glu	(-)
TNF-153L	153rd	Ile→Leu	(++)
TNF-115L-Ser67			(++)
TNF-115LΔN8-Ser67			(++)
TNF-115LΔN8			(++)

(2) Table 4 shows the isoelectric points and cytotoxic activities of human TNF (as a control) and the various human TNF polypeptide mutants of the invention obtained in Examples given hereinbelow.

The cytotoxic activity was evaluated on mouse L-M cells (ATCC, CCL 1.2) by the method of Yamada et al. [J. Biotechnology, 3, 141 (1985)].

The human TNF polypeptide mutants which were determined to be homogeneous from SDS-polyacrylamide gel electrophoretic analysis [U. K. Laemmli, Nature (London), 227, 680 (1970)] were used in this test.

Table 4

Properties of Human TNF Polypeptide Mutant: Isoelectric point and cytotoxic activity		
Mutant polypeptide	Isoelectric point (pI)	Cytotoxic activity (U/μg)
Human TNF	5.9	2,080
TNF-31T	5.8	12
TNF-32Y	5.9	0.18
TNF-32H	6.1	32
TNF-32D	5.5	1.1
TNF-32S	5.8	1.0
TNF-36V	5.9	122
TNE-115L	5.9	12
TNF-115S	5.8	23
TNF-115D	5.7	6.8
TNF-115G	5.9	37
TNF-117H	6.3	31

(3) Table 5 shows the antitumor activities in vivo of human TNF (as a control) and the various human TNF polypeptide mutants obtained in Examples given hereinbelow. The antitumor activity was evaluated as follows:-

Meth A sarcoma cells (2×10^5) were transplanted into the abdominal skin of BALB/c female mice (8 week old). Seven days after the transplantation, the polypeptide was administered once intravenously. The tumor necrotizing response was evaluated 24 hours after the administration by the evaluation standards of Carswell et al. [Proc. Natl. Acad. Sci. USA, 72, 3666 (1975)].

As shown in Table 5, the correlation between the cytotoxic activity in vitro and the antitumor activity in vivo against the transplanted tumor is scarce.

However, some human TNF polypeptide mutants, for example human TNF polypeptide mutant in which the 32nd or 115th amino acid from the N-terminus is replaced, show strong in vivo antitumor activity as compared with their in vitro cytotoxic activity, and have low lethal toxicity.

Table 5

Antitumor Effect of Human TNF Polypeptide Mutant on Meth A Sarcoma Transplanted in Mice:

Polypeptide: dose (unit)/mouse	Necrotic Response -	+	++	+++	Inhibition of Tumor Growth (%)
Human TNF:					
600	1	5	0	0	55
2,000	0	4	2	0	80
6,000	0	0	6	0	87
20,000	0	0	0	6	100
TNF-31T:					
35	7	0	0	0	49
115	2	5	0	0	79
TNF-32Y:					
5	4	3	0	0	26
18	3	4	0	0	46
TNF-32D:					
11	4	0	0	0	63
33	0	5	0	0	82
110	0	1	3	1	81
TNF-32S:					
10	5	0	0	0	17
30	1	4	0	0	68
100	0	1	4	0	97
TNF-32H:					
32	4	1	0	0	45
96	1	4	0	0	73
320	0	3	2	0	100

- to be continued -

Table 5 (continued)

Polypeptide: dose (unit)/mouse	Necrotic Response				Inhibition of Tumor Growth (%)
	-	+	++	+++	
TNF-36V:					
120	2	4	0	0	49
370	0	4	2	0	83
1,200	0	0	2	4	93
3,700	0	0	0	6	93
TNF-115L:					
12	5	1	0	0	70
35	2	4	0	0	73
117	0	5	1	0	78
350	0	2	4	0	91
1,170	0	0	5	1	93
TNF-115S:					
23	3	3	0	0	74
69	0	4	2	0	81
230	0	2	4	0	91
690	0	0	5	1	84
2,300	0	0	1	5	100

- to be continued -

Table 5 (continued)

Polypeptide: dose (unit)/mouse	Necrotic Response -	+	++	+++	Inhibition of Tumor Growth (%)
TNF-115D:					
68	0	5	0	0	57
204	0	2	3	0	100
TNF-115G:					
110	0	5	0	0	68
370	0	1	4	0	98
1,100	0	0	1	4	100
TNF-117H:					
31	4	3	0	0	51
92	1	6	0	0	88
310	0	0	7	0	93

4) Several human TNF polypeptide mutants of this invention as well as human TNF were tested for pyrogenicity in rabbit. The results are shown in Table 6.

The pyrogenicity test was carried out by administering the polypeptide intravenously to rabbit, and observing change in the rectal temperature for 4 hours after the administration. The results are expressed as follows:

(-): a rectal temperarure rise of not more than 0.4 • C

(+): a rectal temperature rise of 0.5 to 0.9 • C

(+ +): a rectal temperature rise of 1.0 • C or more

Table 6

Pyrogenicity of Human TNF Polypeptide Mutant in Rabbit:		
Polypeptide:	dose (μg/kg)	Pyrogenicity
Human TNF:	0.50	(+)
TNF-32Y:	0.56	(-)
	5.6	(-)
	56	(-)
TNF-32H:	0.50	(-)
	5.0	(-)
	50	(-)
TNF-115L:	0.67	(-)
	6.7	(-)
	67	(+)
TNF-115S:	0.52	(-)
	5.2	(+)
	52	(+)
TNF-117H:	0.52	(-)
	5.2	(-)
	52	(+)

(5) The effect of the polypeptide mutant (TMF-115L) on the blood pressure was tested by administering into the tail vein of SHR/NCrj male rats (body weight 264 to 304 g; Nippon Charles River Co., Ltd.), and measuring the systolic blood pressure of the rats without anesthesia by means of an arterial pressure measuring device for rats (Model KN-209, made by Natsume Seisakusho). As a control human TNF was administered as well.

The results are shown in Table 7.

Table 7

Effect of TNF-115L on Blood Pressure in Rats			
Polypeptide dosage (μg/kg)	Changes in Blood Pressure (mean±SD) (hours after Administration)		
	before	5 hours	24 hours
Human TNF:			
100	193±3.1 mmHg	183±2.0 mmHg	170±3.2 mmHg
TNF-115L:			
100	189±3.0 mmHg	195±2.5 mmHg	191±2.6 mmHg
1,000	189±3.0	178±2.7	184±2.2
5,000	190±1.5	186±2.1	189±2.5
10,000	191±2.1	185±2.4	188±3.6

For formulating human TNF polypeptide mutants of this invention, they may be in the form of a solution or a lyophilized product. From the standpoint of long-term stability, they are desirably in the form of lyophilized products. It is preferred to add vehicles or stabilizers to the preparations. Examples of the stabilizers include albumin, globulin, gelatin, protamine, protamine salts, glucose, galactose, xylose, mannitol, glucuronic acid, trehalose, dextran, hydroxyethyl starch, and nonionic surface-active agents (such as polyoxyethylene fatty acid esters, polyoxyethylene alkyl ethers, polyoxyethylene alkyl phenyl ethers, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene glycerin fatty acid esters, polyoxyethylene hardened castor oil, polyoxyethyene castor oil, polyoxyethylene polyoxypropylene alkyl ethers, polyoxyethylene polyoxypropylene block copolymer, sorbitan fatty acid esters, sucrose fatty acid esters and

glycerin fatty acid esters).

The several human TNF polypeptide mutants of this invention are especially useful as antitumor agents as shown above.

Such polypeptide preparations are preferably administered parenterally or topically. Parenteral routes such as intraveous and intramuscular routes are used when tumor cells extend over a wide range or metastasize, or when prevention of metastasis is intended. Against local tumor tissues, direct intratumor administration is preferred. The dosage varies depending upon the type of human TNF polypeptide mutants and the type and size of tumors, the condition of the patient and the route of administration. For example, in case of TNF-115L, it is 1×10^3 to 1×10^8 units (LM)/kg, preferably 1×10^4 to 1×10^7 units (LM)/kg.

The following examples illustrate the present invention more specifically. It should be understood however that other human TNF polypeptide mutants in accordance with this invention can also be produced by similar methods, and the invention is in no way limited to these examples.

Example 1

Production of Human TNF Polypeptide Mutant TNF-32Y:-

(1) Construction of an expression plasmid

An expression plasmid (pHNY-32) for producing a polypeptide consisting of 155 amino acids corresponding to the sequence from amino acid No. 1 to No. 155 in Table 9 attached, referred to as TNF-32Y, was constructed as illustrated in Figures 2 and 3.

A cloned cDNA encoding human TNF was isolated by digestion with restriction endonuclease PstI from the recombinant plasmid pHTNF13 prepared according to the method described in European Patent Publication No. 155549.

The cloned cDNA was further digested with restriction endonucleases AvaI and HindIII to isolate a DNA fragment containing most of the coding region for the human TNF polypeptide. The isolated DNA fragment is referred to as TNF-DNA fragment.

The TNF-DNA fragment was about 600 bp in size containing the base sequence corresponding to the downstream region from base No. 250 in Table 8. Its full base sequence was reported by Yamada et al. [J. Biotechnology, 3, 141 (1985)].

The TNF-DNA fragment was further digested with restriction endonucleases HpaII and BglI to cut it into three DNA fragments and they were isolated. These DNA fragments had the sequences corresponding to the region from base No. 250 to No. 321, the region from base No. 322 to 337 and the downstream region from base No. 338 in Table 8, respectively. These DNA fragments were named DNA-1 fragment, DNA-2 fragment and DNA-3 fragment, respectively.

Then, the DNA-1 and DNA-3 fragments were combined by using T4 DNA ligase with the following chemically synthesized oligodeoxyribonucleotide adapter [a].

```
5'-CGGGCCTATGCCCTCC-3'          [a]
  3'-CCGGATACGGG-5'
```

The ligated DNA fragment is referred to as NY-DNA fragment. The NY-DNA fragment was sequentially ligated with the following two chemically synthesized oligodeoxyribonucleotide adapters, [b] and [c].

```
5'-AACTAGTACGCAAGTTCACGTAAGGAGGTTATC-3'      [b]
3'-TTGATCATGCGTTCAAGTGCATTCCTCCAATAGCTA-5'
```

and

```
5'-GATTATGTCATCTTCTCGAACC-3'          [c]
   3'-ATACAGTAGAAGAGCTTGGGGCT-5'
```

The resulting DNA fragment is referred to as Peptide coding-DNA fragment.

A DNA fragment (about 380 bp in size) containing the trp promoter region was isolated from a plasmid pCT-1 [M. Ikehara et al., Proc. Natl. Acad. Sci., USA, 81, 5956 (1984)] by double digestion with restriction endonucleases HpaI and AatII. The base sequence of the trp promoter region of the above 380 bp-DNA fragment was reported by Bennett et al. [J. Mol. Biol., 121, 113 (1978)]. The above 380 bp-DNA fragment was ligated with the Peptide coding-DNA fragment prepared as above. The ligated DNA fragment was referred to as Promoter-Peptide coding-DNA fragment.

Separately, a plasmid pBR322 was digested with restriction endonucleases AvaI and PvuII, and the resulting larger DNA fragment (about 3.7 kbp in size) was isolated by 0.7% agarose gel electrophoresis. After filling-in its cohesive ends to blunt ends with E. coli DNA polymerase I (Klenow fragment) and four kinds of deoxyribonucleotide triphosphates (dGTP, dATP, dTTP and dCTP), both ends were ligated by T4 DNA ligase to construct a new plasmid, which is designated pBRS6.

The plasmid pBRS6 was cleaved with restriction endonucleases AatII and HindIII into two DNA fragments. The larger DNA fragment (about 3.6 kbp in size) was isolated and ligated by T4 DNA ligase with the Promoter-Peptide coding-DNA fragment prepared as above in order to construct an expression plasmid pHNY-32.

(2) Production of TNF-32Y

The expression plasmid pHNY-32 was introduced into E. coli HB101 by the conventional method [S. N. Cohen et al., Proc. Natl. Acad. Sci., USA, 69, 2110 (1972)].

The transformant (HB101/pHNY-32) was cultivated at 37 • C overnight in LB broth (composition: 1% trypton, 0.5% yeast extract, 1% NaCl, pH 7.5]. The culture was inoculated in 10-volumes of modified M9 medium [composition: 1.5% $Na_2HPO_4 \cdot 12H_2O$, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 2 mg/liter of vitamine Bl, 0.45% casamino acid, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 0.4% glycerol] containing ampicillin at 25 micrograms/ml, at 37 • C for 1 hour.

Then, 3-indoleacrylic acid was added to give a final concentration of 20 micrograms/ml. After the cultivation was further continued for 24 hours, the cells were collected by centrifugation. The cells were suspended in 50 mM Tris-HCl buffer (pH 8) containing 0.1% lysozyme and 30 mM NaCl, and allowed to stand in an ice bath for 30 minutes. After the cell suspension was repeatedly treated by freezing in a dry ice/ethanol bath and thawing at 37 • C, the cell extract was collected by centrifugation.

The cell extract was dialyzed against 20 mM Tris-HCl buffer (pH 7.8), and the dialyzate was centrifuged to obtain a clarified supernatant. The supernatant was applied onto a DEAE-Sepharose CL-6B column (Pharmacia) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.8). After the column was washed with the same buffer to remove non-adsorbed components, the adsorbed polypeptide (TNF-32Y) was eluted with a linear gradient of NaCl concentration from zero to 0.3 M in the same buffer. Each fraction was subjected to SDS-polyacrylamide gel electrophoresis, and the fractions containing polypeptide having a molecular weight of about 17 kilodaltons were collected and pooled.

The pooled fraction was dialyzed against 20 mM Tris-HCl buffer (pH 7.8), and then it was again subjected to DEAE-Sepharose CL-6B column chromatography as above, but the elution was carried out under the elution condition of an easier gradient of NaCl concentration.

The fractions containing the desired polypeptide were collected, pooled and concentrated by ultrafiltration with Diaflo using a YM10 membrane (Amicon).

Finally, the concentrate was subjected to gel filtration on a column of Bio-Gel P-6 (Bio-Rad) using 5 mM phosphate buffered saline as an eluent to obtain purified TNF-32Y.

N-terminal amino acid sequence of the purified TNF-32Y was analyzed by the automated Edman degradation on Protein Sequencer (Applied Biosystems, Model 470A).

As a result, the N-terminal amino acid of TNF-32Y was a serine residue. Namely, a methionine residue due to the translation initiation codon (ATG) was removed from the purified product.

Example 2

Production of Human TNF Polypeptide Mutant TNF-115L:-

(1) Construction of an expression plasmid

An expression plasmid (pHPL-115) for producing a polypeptide consisting of 155 amino acids corresponding to the sequence of the amino acid No. 1 to No. 155 in Table 10 attached, referred to as TNF-

115L, was constructed as illustrated in Figure 4.

The TNF-DNA fragment prepared as mentioned in Example 1-(1) was digested with restriction endonucleases PvuII and TaqI to cut it into four DNA fragments and they were isolated. These DNA fragments had the sequences corresponding to the region from base No. 250 to 369, the region from base No. 370 to 603, the region from base No. 604 to No. 653 and the downstream region from base No. 654 in Table 8, respectively.

These DNA fragments were named DNA-4 fragment, DNA-5 fragment, DNA-6 fragment and DNA-7 fragment, respectively. The DNA-5 fragment was further digested with restriction endonuclease DdeI to isolate a DNA fragment corresponding to the sequence from base No. 370 to No. 554 in Table 8 (referred to as DNA-8 fragment).

The DNA-8 fragment was combined with the DNA-4 fragment, and then ligated with the following two chemically synthesized oligodeoxyribonucleotide adaptors, [d] and [e].

```
5'-TGAGGCCAAGCCCTGGTATGAGCTCAT-3'        [d]
   3'-CCGGTTCGGGACCATACTCGA-5'
```

and

```
5'-CTATCTGGGAGGGGTCTTCCAG-3'        [e]
3'-GTAGATAGACCCTCCCCAGAAGGTC-5'
```

To the ligated DNA fragment, the DNA-6 fragment and the DNA-7 fragment were further ligated by using T4 DNA ligase. The resulting DNA fragment is referred to as PL-DNA fragment.

The expression plasmid pHPL-115 was constructed according to the method as mentioned in Example 1-(1), except for using the PL-DNA fragment instead of the NY-DNA fragment.

(2) Production of TNF-115L

According to the method mentioned in Example 1-(2), the transformant (HB101/pHPL-115) was prepared and cultivated. The desired polypeptide was isolated and purified from the cell extract according to essentially the same method as mentioned in Example 1-(2).

(3) Determination of amino acid sequence

Amino acid sequences of the purified TNF-115L and its peptide fragment were analyzed by the automated Edman degradation on a Protein Sequencer.

The peptide fragment was prepared under the following conditions. Five hundred micrograms of the purified TNF-115L was incubated with 10 micrograms of lysyl endopeptidase (EC 3.4.21.50: Wako Pure Chemical Ind.) in 5 mM Tric-HCl buffer (pH 8) containing 4M urea in a total volume of 0.1 ml. After incubation at 35°C for 15 hours, the resulting digested peptides were isolated by high performance liquid chromatography using a column of SynChropak RP-P300 (250 x 4.6 mm; SynChrom Inc.) under the conditions of a linear gradient elution from 10% to 50% of acetonitrile containing 0.07% trifluoroacetic acid, in 0.1% trifluoroacetic acid, at a flow rate of 1 ml/min for 60 minutes. The elution pattern is shown in Figure. 5. The peptide fragments were isolated from each of fractions of No. 1 to No. 7 in Figure 5 and subjected to analysis of amino acid sequence by the automated Edman degradation method.

The partial amino acid sequence of peptide fragment No. 6 was determined to be Pro-X-Tyr-Glu-Leu-Ile-Tyr-Leu-Gly-Gly-Val-Phe-Gln-Leu-Glu. Mark "X" shows an amino acid which could not be determined by this analysis.

The determined amino acid sequence as above was completely agreed with the sequence from amino acid No. 111 to No. 125 in Table 10.

It was confirmed that the amino acid at the 115th position from the N-terminus of TNF-115L was a leucine residue.

N-terminal amino acid of the purified TNF-115L was a serine residue, indicating that a methionine residue due to the translation initiation codon (ATG) was removed.

Example 3

Production of Other Human TNF Polypeptide Mutants-1:-

(1) Construction of expression plasmids

An expression plasmid for producing a polypeptide consisting of 155 amino acids and having an amino acid sequence corresponding to the sequence from amino acid No. 1 to No. 155 in Table 1, in which an asparagine residue in the 32nd position from the N-terminus was replaced by another amino acid, for example, His, Asp and Ser, was constructed according to the method as mentioned in Example 1-(1), except for using one of the chemically synthesized oligodeoxyribonucleotide adaptors shown below instead of the synthetic adapter [a]:

```
5'-CGGGCCCACGCCCTCC-3'
  3'-CCGGGTGCGGG-5'
                    (for replacement by His),

5'-CGGGCCGATGCCCTCC-3'
  3'-CCGGCTACGGG-5'
                    (for replacement by Asp),
```

or

```
5'-CGGGCCAGCGCCCTCC-3'
  3'-CCGGTCGCGGG-5'
                    (for replacement by Ser).
```

(2) Production of human TNF polypeptide mutant

Each of the expression plasmids obtained in Section (1) was introduced in E. coli HB101 by the conventional method, and the transformant was cultivated according to the method described in Example 1-(2).

The desired polypeptide was purified from the cell extract by essentially the same method as mentioned in Example 1-(2).

There were obtained the followng human TNF polypeptide mutants.

- TNF-32H: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by His.
- TNF-32D: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Asp.
- TNF-32S: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Ser.

Example 4

Production of Other Human TNF Polypeptide Mutants-2:-

(1) Construction of expression plasmids

An expression plasmid for producing a polypeptide consisting of 155 amino acids and having an amino acid sequence corresponding to the sequence from amino acid No. 1 to No. 155 in Table 1, in which a proline residue in the 115th position from the N-terminus was replaced by another amino acid, for example, Ser, Asp and Gly, was constructed according to the method as mentioned in Example 2-(1), except for using one of the chemically synthesized oligodeoxyribonucleotide adapters shown below instead of the

synthetic adapter [d]:

```
5'-TGAGGCCAAGCCCTGGTATGAGTCCAT-3'
   3'-CCGGTTCGGGACCATACTCAG-5'
```

(for replacement by Ser),

```
5'-TGAGGCCAAGCCCTGGTATGAGGACAT-3'
   3'-CCGGTTCGGGACCATACTCCT-5'
```

(for replacement by Asp),

or

```
5'-TGAGGCCAAGCCCTGGTATGAGGGCAT-3'
   3'-CCGGTTCGGGACCATACTCCC-5'
```

(for replacement by Gly).

(2) Production of human TNF polypeptide mutants

Each of the expression plasmids obtained in Section (1) was introduced in E. coli HB101 by the conventional method, and the transformant was cultivated according to the method described in Example 1-(2).

The desired polypeptide was isolated and purified from the cell extract by essentially the same method as described in Example 1-(2).

There were obtained the followng human TNF polypeptide mutants.

- TNF-115S: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Ser.
- TNF-115D: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Asp.
- TNF-115G: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Gly.

Example 5

Production of Human TNF Polypeptide Mutant TNF-117H, referred to as TNF-117H:-

(1) Construction of expression plasmids

An expression plasmid for producing a polypeptide consisting of 155 amino acids and having an amino acid sequence corresponding to the sequence from amino acid No. 1 to No. 155 in Table 1, in which a tyrosine residue in the 117th position from the N-terminus was replaced by another amino acid, for example, His, was constructed according to the method as mentioned in Example 2-(1), except for using a chemically synthesized oligodeoxyribonucleotide adapter shown below instead of the synthetic adapter [e]:

```
   5'-CCATCTGGGAGGGGTCTTCCAG-3'
3'-GTAGGTAGACCCTCCCCAGAAGGTC-5'
```

(2) Production of TNF-117H

The expression plasmid obtained in Section (1) was introduced in E. coli HB101 by the conventional method, and the transformant was cultivated according to the method described in Example 1-(2).

The desired polypeptide was isolated and purified from the cell extract by essentially the same method as mentioned in Example 1-(2).

Example 6

Production of Other Human TNF Polypeptide Mutants-3:-

In accordance with Example 1, expression plasmids for production of the following polypeptides were constructed. Escherichia coli was transformed with the expression plasmids. The transformants were cultured, and the polypeptides were isolated and purified.

TNF-31T: Polypeptide having an amino acid sequence of formula [I] in which 31st Ala was replaced by Thr

TNF-32G: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Gly

Example 7

Production of Other Human TNF Polypeptide Mutants-4:-

In accordance with Example 1, expression plasmids for production of the following polypeptides were constructed. Escherichia coli was transformed with the expression plasmids. The transformants were cultured to produce the polypeptides.

TNF-32A: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Ala

TNF-32C: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Cys

TNF-32I: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Ile

TNF-32R: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Arg

TNF-32T: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Thr

TNF-32V: Polypeptide having an amino acid sequence of formula [I] in which 32nd Asn was replaced by Val

TNF-115A: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Ala

TNF-115F: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Phe

TNF-115N: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Asn

TNF-115Y: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Tyr

TNF-115V: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Val

TNF-115E: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Glu

TNF-115M: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Met

TNF-115I: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Ile

TNF-115K: Polypeptide having an amino acid sequence of formula [I] in which 115th Pro was replaced by Lys

Referential Example 1

Construction of an Expression Plasmid for Producing Human TNF:-

The cloned cDNA encoding human TNF was isolated by digestion with restriction endonuclease PstI, from the recombinant plasmid pHTNF13 prepared according to the method described in European Patent Publication No. 155549.

The cloned cDNA was digested with restriction endonuclease EcoRI to split off part of the non-coding region downstream of the TNF coding region. The resulting DNA fragment (about 1.1 kbp) was inserted into a larger DNA fragment prepared from a plasmid pBR322 by digestion with restriction endonucleases PstI and EcoRI to construct a recombinant plasmid including TNF cDNA and a tetracycline-resistance gene, which was named pHT113.

The recombinant plasmid pHT113 was digested with restriction endonucleases AvaI and SalI to cut it into three fragments (about 0.8 kbp, 1.3 kbp and 2.6 kbp in size). The 1.3 kbp-DNA fragment including most of the coding region for the human TNF and part of tetracycline-resistance gene was isolated (to be referred to as AvaI-SalI fragment). The AvaI-SalI fragment was ligated with the following chemically synthesized oligodeoxyribonucleotide adapter [f].

```
5'-CGATATGTCATCTTCTCGAACC-3'
  3'-TATACAGTAGAAGAGCTTGGGGCT-5'
```
[f]

The resulting DNA fragment is referred to as HTNF-adapter fragment.

Separately, a DNA fragment (35 bp) including part of the trp promoter region was cut out from a plasmid pDR720 [P-L Biochemicals; D. R. Russell, et al., Gene, 20, 231 (1983)] by digesting with restriction endonucleases EcoRI and HpaI. The nucleotide sequence of the isolated 35 bp-DNA fragment is as follows:

```
5'-AATTCCCCTGTTGACAATTAATCATCGAACTAGTT-3'
    3'-GGGGACAACTGTTAATTAGTAGCTTGATCAA-5'
```

The 35 bp-DNA fragment was ligated with a chemically synthesized adapter represented by the following formula:

```
5'-AACTAGTACGCAAGTTCACGTAAAAAGGGTAAT-3'
3'-TTGATCATGCGTTCAAGTGCATTTTTCCCATTAGC-5'
```
[g]

The resulting DNA fragment is referred to as trp promoter fragment.

A plasmid pBR322 was digested with restriction endonucleases EcoRI and SalI, and then the larger DNA fragment (about 3.7 kbp) was isolated.

An expression plasmid for producing human TNF consisting of 155 amino acids corresponding to the amino acid sequence from amino acid No. 79 to No. 233 in Table 8, was constructed by sequential ligation of these three DNA fragments, the HTNF-adapter fragment, the trp promoter fragment and the larger pBR322 fragment (about 3.7 kbp) as illustrated in Figure 6.

The expression plasmid was named pHTR91.

In accordance with Example 1-(2), the plasmid was introduced into Escherichia coli. The transformant was cultured to produce human TNF.

Table 8

```
                                                    GACCCACGG

-30          -20          -10          -1
 CTCCACCCTCTCTCCCCTGGAAAGGACACC
 1           10           20           30
 ATGAGCACTGAAAGCATGATCCGGGACGTG
 MetSerThrGluSerMetIleArgAspVal
                                                          10
             40           50           60
 GAGCTGGCCGAGGAGGCGCTCCCCAAGAAG
 GluLeuAlaGluGluAlaLeuProLysLys
                                                          20
              70           80           90
 ACAGGGGGGCCCCAGGGCTCCAGGCGGTGC
 ThrGlyGlyProGlnGlySerArgArgCys
                                                          30
             100          110          120
 TTGTTCCTCAGCCTCTTCTCCTTCCTGATC
 LeuPheLeuSerLeuPheSerPheLeuIle
                                                          40
             130          140          150
 GTGGCAGGCGCCACCACGCTCTTCTGCCTG
 ValAlaGlyAlaThrThrLeuPheCysLeu
                                                          50
             160          170          180
 CTGCACTTTGGAGTGATCGGCCCCCAGAGG
 LeuHisPheGlyValIleGlyProGlnArg
                                                          60
             190          200          210
 GAAGAGTTCCCCAGGGACCTCTCTCTAATC
 GluGluPheProArgAspLeuSerLeuIle
                                                          70
             220          230          240
 AGCCCTCTGGCCCAGGCAGTCAGATCATCT
 SerProLeuAlaGlnAlaValArgSerSer
                                                          80
```

- to be continued -

Table 8 (continued)

```
          250         260         270
TCTCGAACCCCGAGTGACAAGCCTGTAGCC
SerArgThrProSerAspLysProValAla
                                    90
          280         290         300
CATGTTGTAGCAAACCCTCAAGCTGAGGGG
HisValValAlaAsnProGlnAlaGluGly
                                   100
          310         320         330
CAGCTCCAGTGGCTGAACCGCCGGGCCAAT
GlnLeuGlnTrpLeuAsnArgArgAlaAsn
                                   110
          340         350         360
GCCCTCCTGGCCAATGGCGTGGAGCTGAGA
AlaLeuLeuAlaAsnGlyValGluLeuArg
                                   120
          370         380         390
GATAACCAGCTGGTGGTGCCATCAGAGGGC
AspAsnGlnLeuValValProSerGluGly
                                   130
          400         410         420
CTGTACCTCATCTACTCCCAGGTCCTCTTC
LeuTryLeuIleTyrSerGlnValLeuPhe
                                   140
          430         440         450
AAGGGCCAAGGCTGCCCCTCCACCCATGTG
LysGlyGlnGlyCysProSerThrHisVal
                                   150
          460         470         480
CTCCTCACCCACACCATCAGCCGCATCGCC
LeuLeuThrHisThrIleSerArgIleAla
                                   160
          490         500         510
GTCTCCTACCAGACCAAGGTCAACCTCCTC
ValSerTyrGlnThrLysValAsnLeuLeu
                                   170
```

- to be continued -

Table 8 (continued)

```
          520         530         540
TCTGCCATCAAGAGCCCCTGCCAGAGGGAG
SerAlaIleLysSerProCysGlnArgGlu
                                    180
          550         560         570
ACCCCAGAGGGGGCTGAGGCCAAGCCCTGG
ThrProGluGlyAlaGluAlaLysProTrp
                                    190
          580         590         600
TATGAGCCCATCTATCTGGGAGGGGTCTTC
TyrGluProIleTyrLeuGlyGlyValPhe
                                    200
          610         620         630
CAGCTGGAGAAGGGTGACCGACTCAGCGCT
GlnLeuGluLysGlyAspArgLeuSerAla
                                    210
          640         650         660
GAGATCAATCGGCCCGACTATCTCGACTTT
GluIleAsnArgProAspTyrLeuAspPhe
                                    220
          670         680         690
GCCGAGTCTGGGCAGGTCTACTTTGGGATC
AlaGluSerGlyGlnValTyrPheGlyIle
                                    230
          700         710         720
ATTGCCCTGTGAGGAGGACGAACATCCAAC
IleAlaLeu
          730         740
CTTCCCAAACGCCTCCCCTGC
```

Table 9

```
1         10         20         30
TCATCTTCTCGAACCCCGAGTGACAAGCCT
SerSerSerArgThrProSerAspLysPro
 1                             10
          40         50         60
GTAGCCCATGTTGTAGCAAACCCTCAAGCT
ValAlaHisValValAlaAsnProGlnAla
                               20
          70         80         90
GAGGGGCAGCTCCAGTGGCTGAACCGCCGG
GluGlyGlnLeuGlnTrpLeuAsnArgArg
                               30
         100        110        120
GCCTATGCCCTCCTGGCCAATGGCGTGGAG
AlaTyrAlaLeuLeuAlaAsnGlyValGlu
                               40
         130        140        150
CTGAGAGATAACCAGCTGGTGGTGCCATCA
LeuArgAspAsnGlnLeuValValProSer
                               50
         160        170        180
GAGGGCCTGTACCTCATCTACTCCCAGGTC
GluGlyLeuTryLeuIleTyrSerGlnVal
                               60
         190        200        210
CTCTTCAAGGGCCAAGGCTGCCCCTCCACC
LeuPheLysGlyGlnGlyCysProSerThr
                               70
         220        230        240
CATGTGCTCCTCACCCACACCATCAGCCGC
HisValLeuLeuThrHisThrIleSerArg
                               80
         250        260        270
ATCGCCGTCTCCTACCAGACCAAGGTCAAC
IleAlaValSerTyrGlnThrLysValAsn
                               90
```

- to be continued -

Table 9 (continued)

```
        280         290         300
CTCCTCTCTGCCATCAAGAGCCCCTGCCAG
LeuLeuSerAlaIleLysSerProCysGln
                                100
        310         320         330
AGGGAGACCCCAGAGGGGGCTGAGGCCAAG
ArgGluThrProGluGlyAlaGluAlaLys
                                110
        340         350         360
CCCTGGTATGAGCCCATCTATCTGGGAGGG
ProTrpTyrGluProIleTyrLeuGlyGly
                                120
        370         380         390
GTCTTCCAGCTGGAGAAGGGTGACCGACTC
ValPheGlnLeuGluLysGlyAspArgLeu
                                130
        400         410         420
AGCGCTGAGATCAATCGGCCCGACTATCTC
SerAlaGluIleAsnArgProAspTyrLeu
                                140
        430         440         450
GACTTTGCCGAGTCTGGGCAGGTCTACTTT
AspPheAlaGluSerGlyGlnValTyrPhe
                                150
        460
GGGATCATTGCCCTGTGA
GlyIleIleAlaLeu***
            155
```

Table 10

```
1          10          20          30
TCATCTTCTCGAACCCCGAGTGACAAGCCT
SerSerSerArgThrProSerAspLysPro
 1                              10
          40          50          60
GTAGCCCATGTTGTAGCAAACCCTCAAGCT
ValAlaHisValValAlaAsnProGlnAla
                                  20
          70          80          90
GAGGGGCAGCTCCAGTGGCTGAACCGCCGG
GluGlyGlnLeuGlnTrpLeuAsnArgArg
                                  30
         100         110         120
GCCTATGCCCTCCTGGCCAATGGCGTGGAG
AlaTyrAlaLeuLeuAlaAsnGlyValGlu
                                  40
         130         140         150
CTGAGAGATAACCAGCTGGTGGTGCCATCA
LeuArgAspAsnGlnLeuValValProSer
                                  50
         160         170         180
GAGGGCCTGTACCTCATCTACTCCCAGGTC
GluGlyLeuTryLeuIleTyrSerGlnVal
                                  60
         190         200         210
CTCTTCAAGGGCCAAGGCTGCCCCTCCACC
LeuPheLysGlyGlnGlyCysProSerThr
                                  70
         220         230         240
CATGTGCTCCTCACCCACACCATCAGCCGC
HisValLeuLeuThrHisThrIleSerArg
                                  80
         250         260         270
ATCGCCGTCTCCTACCAGACCAAGGTCAAC
IleAlaValSerTyrGlnThrLysValAsn
                                  90
```

- to be continued -

Table 10 (continued)

```
          280         290         300
CTCCTCTCTGCCATCAAGAGCCCCTGCCAG
LeuLeuSerAlaIleLysSerProCysGln
                                  100
          310         320         330
AGGGAGACCCCAGAGGGGGCTGAGGCCAAG
ArgGluThrProGluGlyAlaGluAlaLys
                                  110
          340         350         360
CCCTGGTATGAGCTCATCTATCTGGGAGGG
ProTrpTyrGluLeuIleTyrLeuGlyGly
                                  120
          370         380         390
GTCTTCCAGCTGGAGAAGGGTGACCGACTC
ValPheGlnLeuGluLysGlyAspArgLeu
                                  130
          400         410         420
AGCGCTGAGATCAATCGGCCCGACTATCTC
SerAlaGluIleAsnArgProAspTyrLeu
                                  140
          430         440         450
GACTTTGCCGAGTCTGGGCAGGTCTACTTT
AspPheAlaGluSerGlyGlnValTyrPhe
                                  150
          460
GGGATCATTGCCCTGTGA
GlyIleIleAlaLeu***
                155
```

Claims

1. A polypeptide having an amino acid sequence represented by formula [I]

Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro
Val Ala His Val Val Ala Asn Pro Gln Ala
Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
Ala Asn Ala Leu Leu Ala Asn Gly Val Glu
Leu Arg Asp Asn Gln Leu Val Val Pro Ser
Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val
Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr
His Val Leu Leu Thr His Thr Ile Ser Arg
Ile Ala Val Ser Tyr Gln Thr Lys Val Asn
Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln
Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly
Val Phe Gln Leu Glu Lys Gly Asp Arg Leu
Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu
Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe
Gly Ile Ile Ala Leu ... [I]

in which at least one of
the following replacements of amino acids is effected:
31st Ala by Thr,
32nd Asn by Ala, Cys, Asp, His, Ile, Arg, Ser, Thr, Val or Tyr,
115th Pro by Ser, Ala, Phe, Asn, Gly, Tyr, Val, Glu, Met, Ile, Asp, Trp, Leu or Lys, and
117th Tyr by His.

2. A polypeptide according to claim 1 wherein in the amino acid sequence of formula [I], at least one of the following replacements of amino acids is effected:
32nd Asn by Tyr, His, Asp or Ser,
115th Pro by Leu, Ser, Asp or Gly, and
117th Tyr by His.

3. A polypeptide according to claim 1 wherein in the amino acid sequence of formula [I], 32nd Asn is replaced by Tyr.

4. A polypeptide of claim 1 wherein in the amino acid sequence of formula [I], 115th Pro is replaced by Leu.

5. A DNA having a base sequence encoding a polypeptide according to any one of claims 1 to 4.

Patentansprüche

1. Polypeptid mit einer Aminosäuresequenz der Formel [I],

Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro
Val Ala His Val Val Ala Asn Pro Gln Ala
Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
Ala Asn Ala Leu Leu Ala Asn Gly Val Glu
Leu Arg Asp Asn Gln Leu Val Val Pro Ser
Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val
Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr
His Val Leu Leu Thr His Thr Ile Ser Arg
Ile Ala Val Ser Tyr Gln Thr Lys Val Asn
Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln
Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly
Val Phe Gln Leu Glu Lys Gly Asp Arg Leu
Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu
Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe
Gly Ile Ile Ala Leu ... [I]

worin mindestens einer der folgenden Aminosäureaustausche durchgeführt ist:
31ste Ala durch Thr,
32ste Asn durch Ala, Cys, Asp, His, Ile, Arg, Ser, Thr, Val oder Tyr,
115te Pro durch Ser, Ala, Phe, Asn, Gly, Tyr, Val, Glu, Met, Ile, Asp, Trp, Leu oder Lys, und
117te Tyr durch His.

2. Polypeptid nach Anspruch 1, worin in der Aminosäuresequenz der Formel [I] mindestens einer der folgenden Aminosäureaustausche durchgeführt ist:
32ste Asn durch Tyr, His, Asp oder Ser,
115te Pro durch Leu, Ser, Asp oder Gly, und
117te Tyr durch His.

3. Polypeptid nach Anspruch 1, worin in der Aminosäuresequenz der Formel [I] das 32ste Asn durch Tyr ersetzt ist.

4. Polypeptid nach Anspruch 1, worin in der Aminosäuresequenz der Formel [I] das 115te Pro durch Leu ersetzt ist.

5. DNA mit einer Basensequenz, die ein Polypeptid nach einem der Ansprüche 1 bis 4 codiert.

Revendications

1. Polypeptide ayant une séquence d'acides aminés représentée par la formule [I]

Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro
Val Ala His Val Val Ala Asn Pro Gln Ala
Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
Ala Asn Ala Leu Leu Ala Asn Gly Val Glu
Leu Arg Asp Asn Gln Leu Val Val Pro Ser
Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val
Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr
His Val Leu Leu Thr His Thr Ile Ser Arg
Ile Ala Val Ser Tyr Gln Thr Lys Val Asn
Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln
Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly
Val Phe Gln Leu Glu Lys Gly Asp Arg Leu
Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu
Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe
Gly Ile Ile Ala Leu ... [I]

dans laquelle est effectué au moins l'un des remplacements suivants d'acides aminés :
31ème : Ala par Thr,
32ème : Asn par Ala, Cys, Asp, His, Ile, Arg, Ser, Thr, Val ou Tyr,
115ème : Pro par Ser, Ala, Phe, Asn, Gly, Tyr, Val, Glu, Met, Ile, Asp, Trp, Leu ou Lys, et
117ème : Tyr par His.

2. Polypeptide selon la revendication 1, dans lequel, dans la séquence d'acides aminés de formule [I], au moins l'un des remplacements suivants d'acides aminés est effectué :
32ème : Asn par Tyr, His, Asp ou Ser,
115ème : Pro par Leu, Ser, Asp ou Gly, et
117ème : Tyr par His.

3. Polypeptide selon la revendication 1, dans lequel, dans la séquence d'acides aminés de formule [I], le 32ème acide aminé Asn est remplacé par Tyr.

4. Polypeptide selon la revendication 1 dans lequel, dans la séquence d'acides aminés de formule [I], le 115ème acide aminé Pro est remplacé par Leu.

5. ADN ayant une séquence de bases codant pour un polypeptide selon l'une quelconque des revendications 1 à 4.

FIG. 1

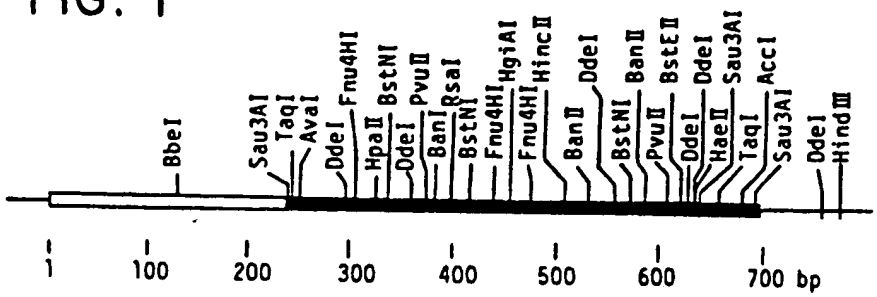

BbeI
Sau3AI
TaqI
AvaI
DdeI
Fnu4HI
HpaII
BstNI
PvuII
DdeI
BanI
RsaI
BstNI
Fnu4HI
HgiAI
Fnu4HI
HincII
BanII
DdeI
BstNI
BanII
PvuII
BstEII
DdeI
DdeI
HaeII
Sau3AI
TaqI
AccI
Sau3AI
DdeI
HindIII
1
100
200
300
400
500
600
700 bp

FIG. 3

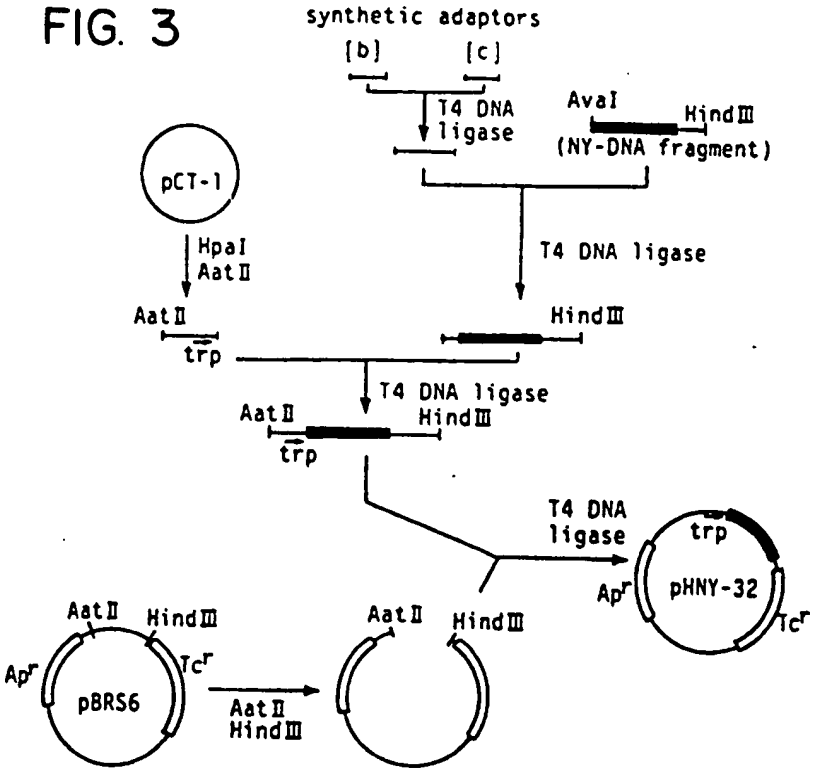

synthetic adaptors
[b]
[c]
T4 DNA ligase
AvaI
HindIII
(NY-DNA fragment)
pCT-1
HpaI
AatII
T4 DNA ligase
AatII
HindIII
trp
T4 DNA ligase
AatII
HindIII
trp
T4 DNA ligase
trp
pHNY-32
Ap^r
Tc^r
AatII
HindIII
Ap^r
pBRS6
Tc^r
AatII
HindIII
AatII
HindIII

FIG. 2

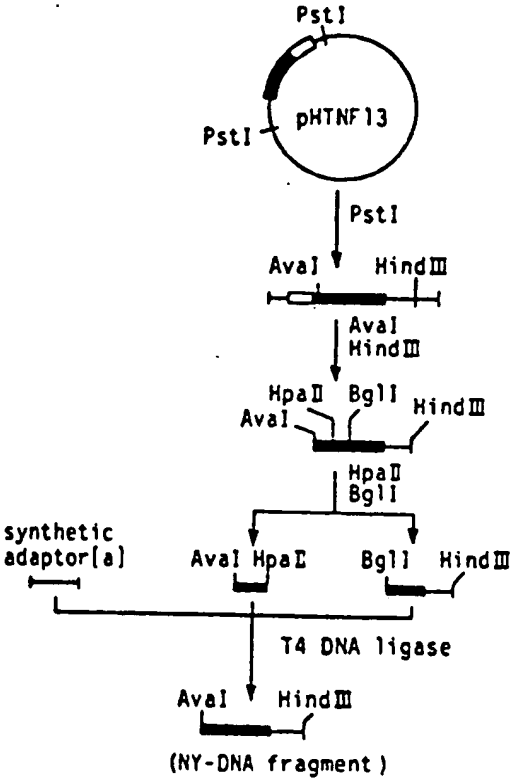

PstI
pHTNF13
PstI
PstI
AvaI
HindIII
AvaI
HindIII
HpaII
BglI
AvaI
HindIII
HpaII
BglI
synthetic
adaptor[a]
AvaI
HpaII
BglI
HindIII
T4 DNA ligase
AvaI
HindIII
(NY-DNA fragment)

FIG. 4

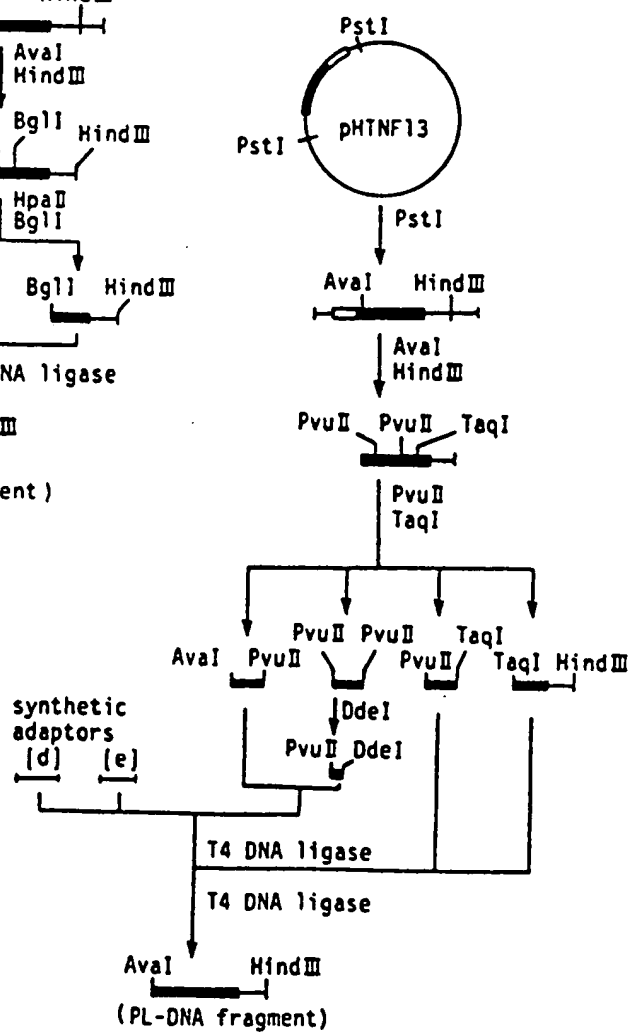

PstI
pHTNF13
PstI
PstI
AvaI
HindIII
AvaI
HindIII
PvuII
PvuII
TaqI
PvuII
TaqI
AvaI
PvuII
PvuII
PvuII
TaqI
HindIII
DdeI
PvuII
DdeI
synthetic
adaptors
[d]
[e]
T4 DNA ligase
T4 DNA ligase
AvaI
HindIII
(PL-DNA fragment)

FIG. 5

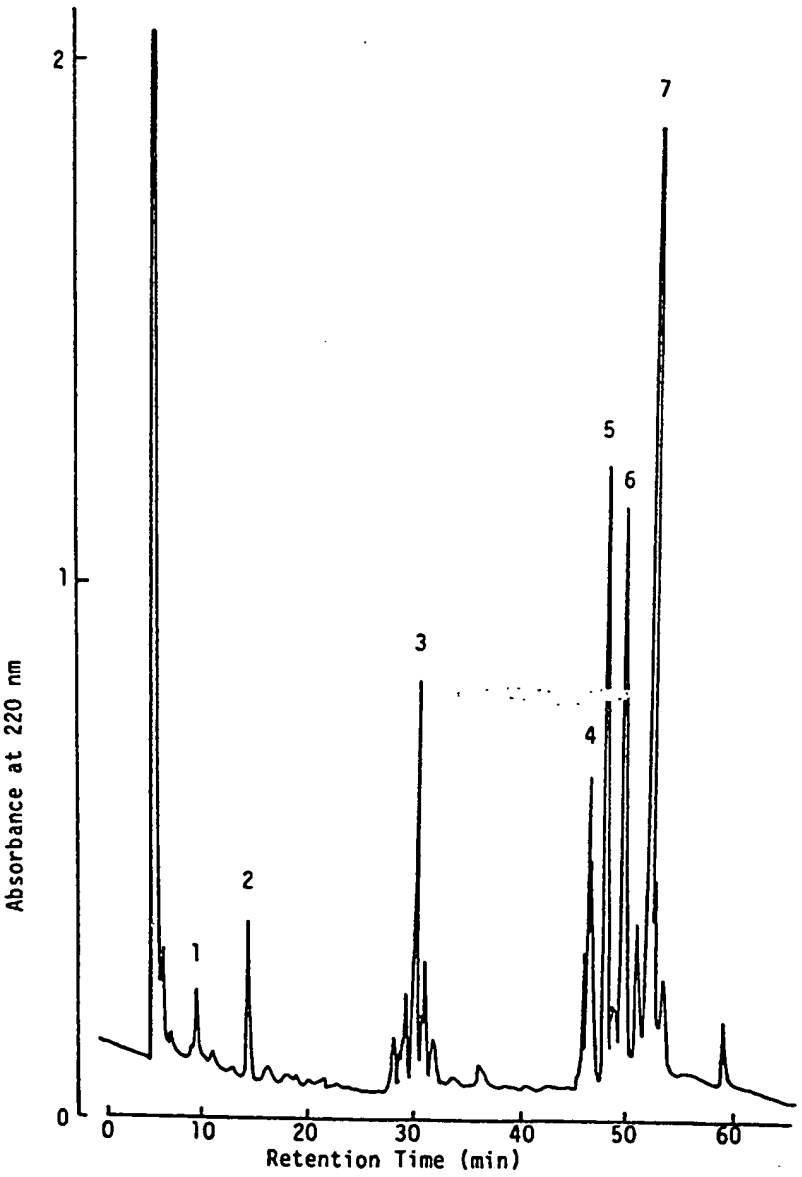
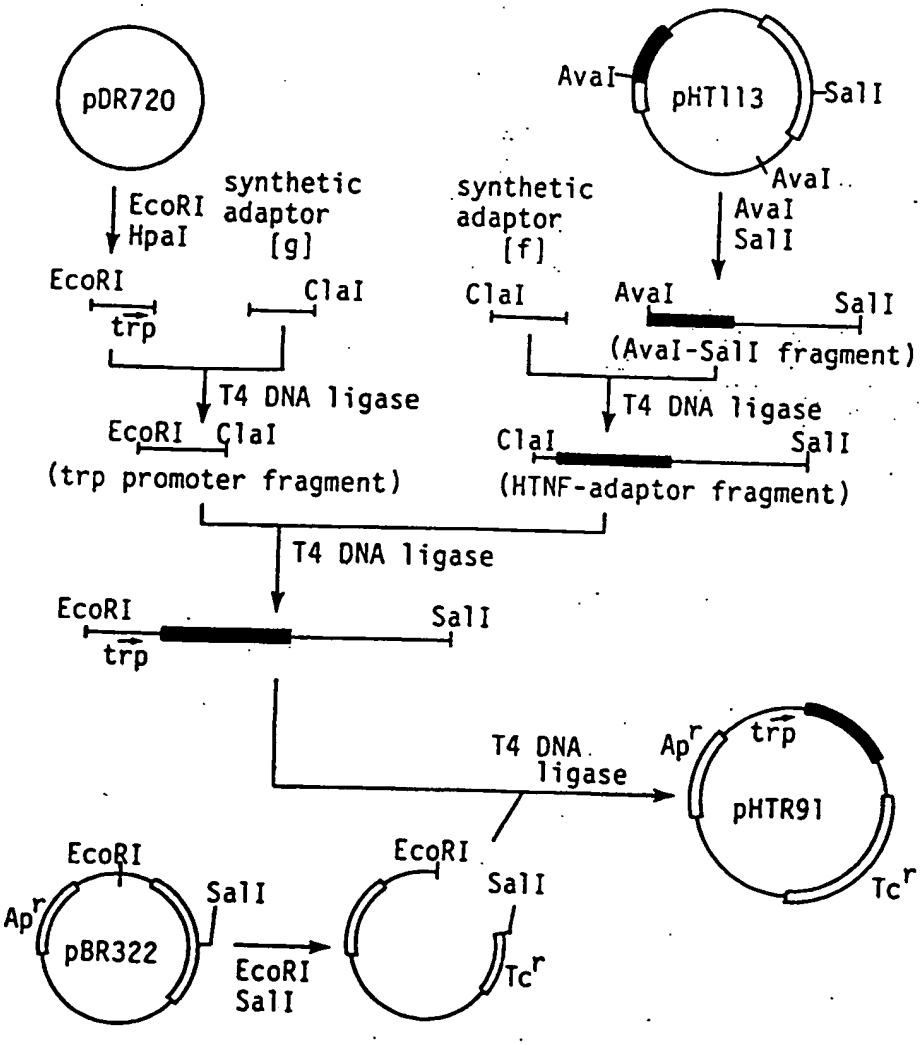

Absorbance at 220 nm
2
1
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
Retention Time (min)

FIG. 6


pDR720
EcoRI
HpaI
synthetic
adaptor
[g]
EcoRI
trp
ClaI
T4 DNA ligase
EcoRI ClaI
(trp promoter fragment)
AvaI
pHT113
SalI
AvaI
synthetic
adaptor
[f]
AvaI
SalI
ClaI
AvaI
SalI
(AvaI-SalI fragment)
T4 DNA ligase
ClaI
SalI
(HTNF-adaptor fragment)
T4 DNA ligase
EcoRI
trp
SalI
T4 DNA
ligase
Ap^r
trp
pHTR91
Tc^r
EcoRI
SalI
Ap^r
pBR322
EcoRI
SalI
EcoRI
SalI
Tc^r